# Rho GTPases regulate distinct aspects of dendritic arbor growth in *Xenopus* central neurons *in vivo*

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**The development and structural plasticity of dendritic arbors are governed by several factors, including synaptic activity, neurotrophins and other growth-regulating molecules. The signal transduction pathways leading to dendritic structural changes are unknown, but likely include cytoskeleton regulatory components. To test whether GTPases regulate dendritic arbor development, we collected time-lapse images of single optic tectal neurons in albino *Xenopus* tadpoles expressing dominant negative or constitutively active forms of Rac, Cdc42 or RhoA. Analysis of images collected at two-hour intervals over eight hours indicated that enhanced Rac activity selectively increased branch additions and retractions, as did Cdc42 to a lesser extent. Activation of endogenous RhoA decreased branch extension without affecting branch additions and retractions, whereas dominant-negative RhoA increased branch extension. Finally, we provide data suggesting that RhoA mediates the promotion of normal dendritic arbor development by NMDA receptor activation.**

The structure of the dendritic arbor critically determines what synaptic inputs a neuron receives and how they are integrated[1]. For instance, a neuron within the visual system whose dendritic arbor has a large tangential spread can receive inputs from more visual afferents, leading to a larger receptive field over which information is processed. Similarly, neurons that extend their dendritic arbors into superficial laminae can receive inputs and process information from afferents in those laminae[2]. Consequently, factors that regulate development and plasticity of the dendritic arbor control both the neuron's structure and function and may ultimately affect circuit properties.

The molecular mechanisms that underlie development of the dendritic arbor are not clear yet. *In vivo* time-lapse imaging permits direct observation of dendritic arbor development. Time-lapse images of optic tectal neurons collected at intervals ranging from minutes to days in living *Xenopus* tadpoles show that dendritic branches are very dynamic during arbor formation[3–6]. The dynamic processes include addition of new branches, retraction of branches, and selective extension or shortening of existing branches. These events can be observed and quantified by collecting repeated images over several hours[3–6]. The data suggest that the net growth of dendritic arbor occurs as a result of several distinct events: emergence of a new branch, selective maintenance of the new branch, and extension of branch length. Each of these events may be individually regulated. Furthermore, it is very likely that machinery controlling the actin cytoskeleton is involved, because cytoskeleton reorganization accompanies structural changes in cells.

Members of the Rho family of small GTPases, Rac, Cdc42 and RhoA, are required components of signal transduction pathways through which extracellular signals cause morphological changes in various cell types[7–9]. Rho GTPases mediate these changes by regulating the cytoskeleton. Investigations of their function have been aided by mutations that result in a constitutively active, GTP-bound form or a dominant-negative, GDP-bound form. Studies of neurite outgrowth from cultured neurons have given us the first insights into the role of the Rho family of GTPases in regulating neuronal process growth[9–11]. Because neurites in cell culture often fail to take on the characteristics of dendrites and axons, few such studies have been able to distinguish effects of Rho GTPases on axonal and dendritic outgrowth. Rac, Cdc42 and RhoA influence dendrite number in dissociated cortical neurons[12]. Given that neuronal arbor elaboration *in vivo* is governed by activity-dependent and activity-independent factors[13], which may operate through GTPases[14], we wanted to determine whether Rho GTPases regulate dendritic arbor formation in the live animal with the normal pattern of synaptic inputs and local environment. Our previous studies showed that NMDA receptor activity is required for the initial phase of dendritic arbor growth in tectal neurons[5,6]. A potential link between synaptic activity and the Rho GTPases remains to be defined.

As opposed to cultured cells, studies in intact animals provide the opportunity to investigate dendritic and axonal development in their normal complex environment. *In vivo* experiments in transgenic flies, worms, *Xenopus* and mice all point to a crucial role of Rac and Cdc42 in axonal growth and target recognition[15–19]. The roles of the Rho GTPases in regulating dendritic arbor structural plasticity *in vivo* are relatively unexplored[20]. Although the dendrites of Purkinje cells in transgenic mice expressing constitutively active Rac in the cerebellum branch normally, dendritic spines are reduced in size and increased in number[16]. In *Xenopus*, retinal ganglion cell dendritic arbor elaboration is inhibited by expression of constitutively active RhoA and constitutively active Cdc42, but promoted by constitutively

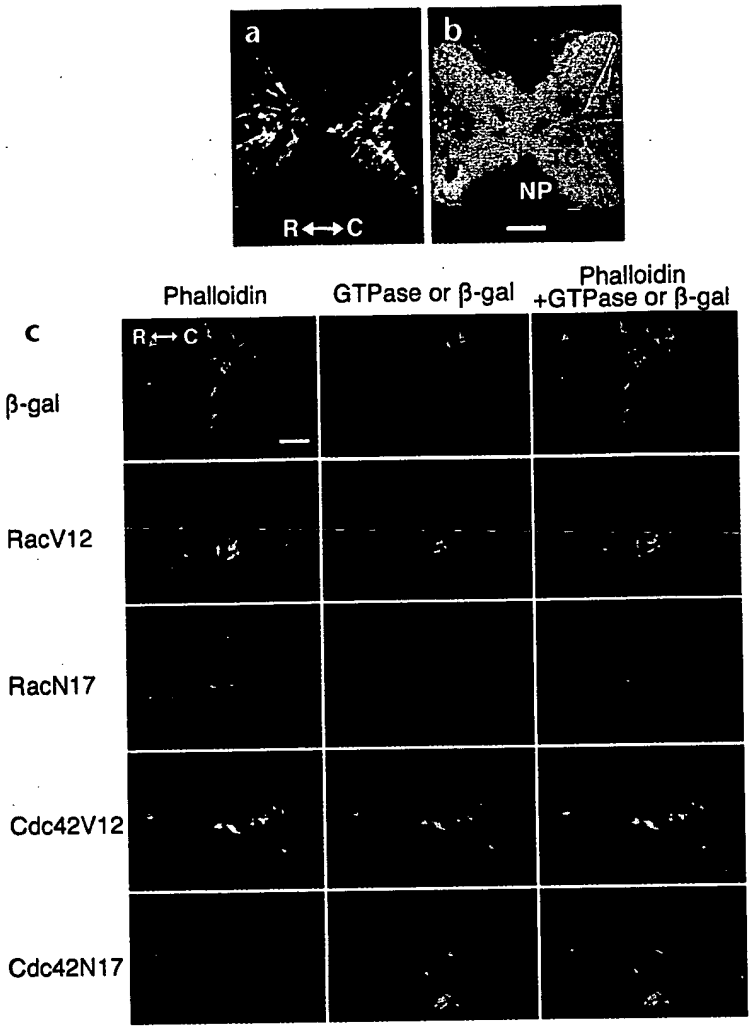


**Fig. 1.** Expression of Rho GTPases and their effect on the actin cytoskeleton. (a) Myc immunostaining shows the distribution of infected, myc-tagged, GTPase-expressing neurons in a horizontal section through the midbrain of a stage 47 tadpole. Most cells near the tectal ventricle (those imaged in later experiments) are infected. (b) Propidium iodide staining of a section neighboring the one shown in (a). Optic tectal cells (TC) are stained and appear gray. The neuropil (NP) region is unstained. (c) Phalloidin staining (left), GTPase expression (middle) in infected neurons, detected by myc immunoreactivity for constitutively active Rac (RacV12), dominant-negative Rac (RacN17) and constitutively active Cdc42 (Cdc42V12) or EGFP for dominant-negative Cdc42 (Cdc42N17), and the overlay of the two images (right) in sections from animals infected at low titer. Constitutively active forms of Rac and Cdc42 specifically increase actin polymerization in GTPase-expressing neurons. Scale bars, 50 μm.

active Rac[18]. In transgenic *Drosophila*, constitutively active and dominant-negative Rac both block axonal growth of sensory neurons, without affecting dendrites, whereas constitutively active Cdc42 inhibits both axonal and dendritic growth[15]. As in the cell culture studies, some of the phenotypic outcomes resulting from expression of Rac and Cdc42 vary between the different studies, likely because of differences in neuron type[15,17]. Nevertheless, these studies provide growing evidence that Rac and Cdc42 can regulate dendritic arbor morphology. These studies do not indicate the potential function of Rho GTPases in dendritic arbor development, nor do they reveal how activity of different GTPases might cooperate during dendrite elaboration.

To address these open issues, we investigated whether Rho GTPases regulate branch dynamics and dendritic arbor growth in optic tectal neurons in live *Xenopus* tadpoles, by collecting *in vivo* time-lapse images of single DiI-labeled tectal neurons. We used vaccinia virus-mediated gene transfer to express constitutively active and dominant-negative forms of RhoA, Rac and Cdc42 in tectal cells. We found that the three Rho GTPases have distinct effects in dendritic arbor development: Rac and Cdc42 regulate dynamic branch additions and retractions, whereas RhoA regulates elongation of existing branches. These results support the idea that dendritic arbor growth occurs through a multi-step process in which Rac and Cdc42 regulate the addition of short branches. RhoA regulates the selective extension of a subset of the added branches.

## Results

### Effects of Rho family GTPases on the actin cytoskeleton

Tectal neurons were infected by ventricular injection of recombinant vaccinia viruses expressing constitutively active and dominant-negative forms of Rho family GTPases. The constitutively active mutants we used were RacV12 and Cdc42V12, and the dominant-negative mutants were RacN17, Cdc42N17 and RhoN19. The GTPases were also tagged with myc or enhanced green fluorescent protein (EGFP; Methods). The myc-tagged GTPases were expressed as IRES (internal ribosome entry site)-EGFP constructs that allowed us to identify infected regions of tectum for DiI labeling. Cryostat sections of infected animals immunostained for the myc-tagged GTPases showed that after injection of high-titer virus, most tectal cells near the brain ventricle were infected (Fig. 1a). Neurons in this region were DiI labeled for imaging experiments described below. Expression of foreign protein was detected starting six hours after injection and was maintained throughout the experiment. Retinal ganglion cells are not infected when virus is injected into the ventricle, so retinal axons do not express foreign protein (Fig. 1), consistent with our previous data[21].

To test whether virally expressed Rho GTPases regulate the actin cytoskeleton in infected tectal neurons, we studied the distribution of polymerized actin using double labeling with phalloidin to visualize polymerized actin and either anti-myc antibody or EGFP to visualize Rho GTPase-expressing cells. The animals were infected with low-titer ($10^6$ pfu) virus to infect only a few tectal cells per animal, so that we could detect the actin filaments in individual GTPase-expressing neurons. In both uninfected animals and those infected with vaccinia virus expressing β-gal, filamentous actin (F-actin) was enriched in the neuropil region, with relatively little F-actin found in the cell body region of the tectum (Fig. 1b and c). Actin polymerization was increased dramatically in neurons expressing constitutively active Rac and constitutively active Cdc42, as indicated by strong phalloidin staining in infected cells (Fig. 1c). In neurons expressing dominant-negative Rac, dominant-negative Cdc42 or dominant-negative RhoA, the F-actin staining pattern was similar to that observed in control

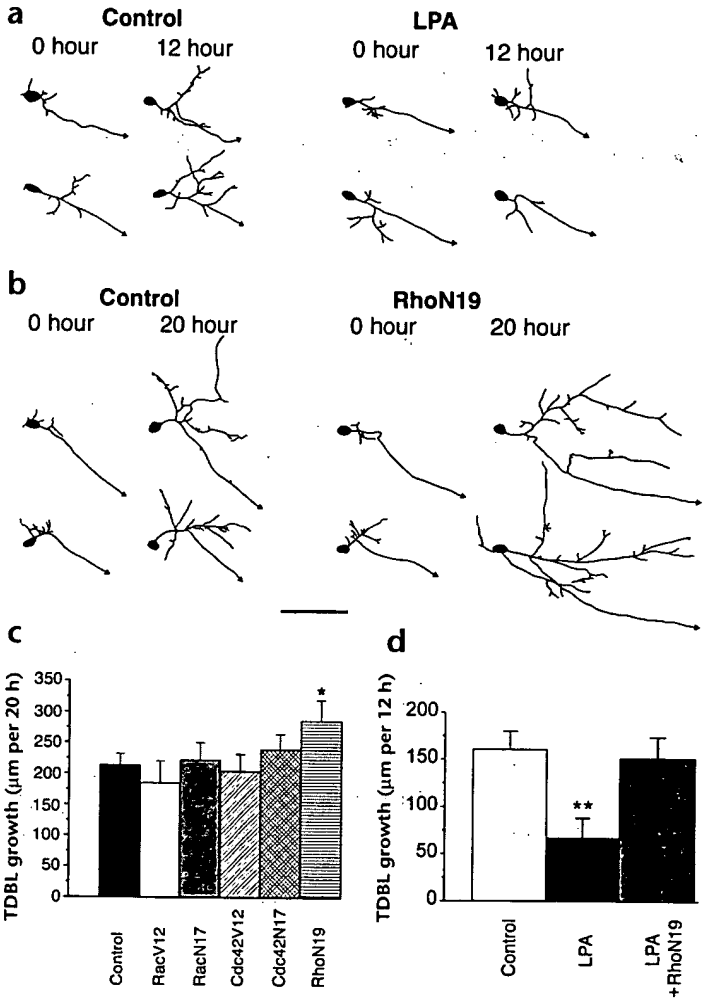


**Fig. 2.** RhoA activity blocks dendritic arbor elaboration. (a) Drawings of 2 representative tectal cells from control and LPA-treated animals imaged over 12 h *in vivo*. (b) Drawings of 2 representative tectal cells from control and dominant-negative RhoA vaccinia virus-infected animals imaged over 20 h. (c) Change in total dendritic branch length (TDBL) over 20 h for neurons imaged from animals infected with various viruses. (d) Change in TDBL over 12 h for neurons from controls, LPA-treated animals and LPA-treated animals expressing dominant-negative RhoA. For each condition, 18–47 cells were analyzed. Scale bar, 50 μm, applies to (a) and (b). $*p < 0.05$; $**p < 0.002$.

cells. F-actin staining was also indistinguishable in lysophosphatidic acid (LPA)-treated animals and control animals, consistent with reports that neuronal cells do not form stress fibers after LPA exposure[22]. These experiments confirm viral expression of the GTPases. They further show that constitutively active Rac and constitutively active Cdc42 caused changes in actin cytoskeleton comparable to those reported previously[7,9]. One possible scenario is that these actin cytoskeleton alterations affect dendritic arbor growth.

In previous experiments, we collected time-lapse images of tectal cell dendrites at three-minute intervals over about an hour, at thirty-minute intervals over two hours, at two-hour intervals over eight hours and at daily intervals over five days[3–6]. These experiments demonstrated that short branches are continually added and retracted from the arbor and that arbor growth results from selective stabilization of a small fraction of the newly added branches and their subsequent elongation.

To assess the effects of Rho GTPases on branch additions and retractions (which we call branch dynamics) as well as branch elongation, we chose an imaging protocol that permits quantitation of parameters relating to branch dynamics and net dendritic arbor growth. One day after injecting virus, we labeled single tectal cells with DiI and collected an initial image of the labeled tectal neurons two hours after DiI labeling. The following day, 12 hours after collecting the first image, we found the same single cell and imaged it at 2-hour intervals over the 8 hours from the 12-hour to 20-hour time points. To determine the effect of GTPase activity on overall dendritic arbor growth, we compared total dendritic branch length at the 0-hour and 20-hour timepoints. An increase in growth rate indicates an increase in branch elongation. To determine the effect of GTPase activity on dendritic arbor dynamics, we compared branch additions and retractions in sequential two-hour time points over eight hours. This imaging interval permits us to follow the fate of every branch we image within the arbor over time[3–5]. This protocol underestimates the rates of branch additions and retractions, because branches are continuously added and retracted within the two-hour intervals. Nevertheless, it does provide a relative measure of arbor dynamics between control and experimental conditions in the same neurons where we can also quantify arbor growth due to branch elongation (see Methods for details).

## Activated RhoA inhibits dendritic branch extension

Expression of dominant-negative RhoA significantly increased arbor growth rate compared to control cells ($p < 0.05$, Fig. 2; growth rate in control cells, 213.7 ± 18.8 μm per 20 h; in neurons from animals infected with dominant-negative RhoA vaccinia virus, 286.0 ± 33.2 μm per 20 h). We used LPA (10 μM in rearing solution) to activate endogenous RhoA because we were not able to generate a virus expressing constitutively active RhoA, most likely because the protein is toxic. LPA has been shown to activate RhoA specifically in neuronal cell lines[11]. Activation of RhoA by LPA treatment significantly reduced dendritic arbor growth rate from the control value of 160.8 ± 19.0 μm per 12 h to 67.4 ± 20.9 μm per 12 h ($p < 0.002$; Fig. 2). To assure that the effect of LPA on growth rate is due to activating RhoA, we showed that the growth-inhibiting effect of LPA could be counteracted by expression of dominant-negative RhoA. Cells from animals infected with dominant-negative RhoA vaccinia virus and treated with LPA had a growth rate of 151.6 ± 22.1 μm per 12 h, comparable to control neurons ($p = 0.85$; Fig. 2). These data indicate that LPA is acting through RhoA to control branch elongation in tectal neurons. In contrast to RhoA, Rac and Cdc42 did not alter dendritic growth rates (Fig. 2). Although RhoA is essential for dendritic branch extension, data shown below indicate that neither LPA nor dominant-negative RhoA changed branch number and branch dynamics (Fig. 3). Together, these data suggest that RhoA regulates the growth of the dendritic arbor by affecting the elongation of pre-existing branches.

## Rac regulates arbor dynamics

To study the effects of Rho family GTPases on dendritic arbor dynamics, we imaged the same neuron every two hours for a total of eight hours. During dendritic arbor formation, the structure

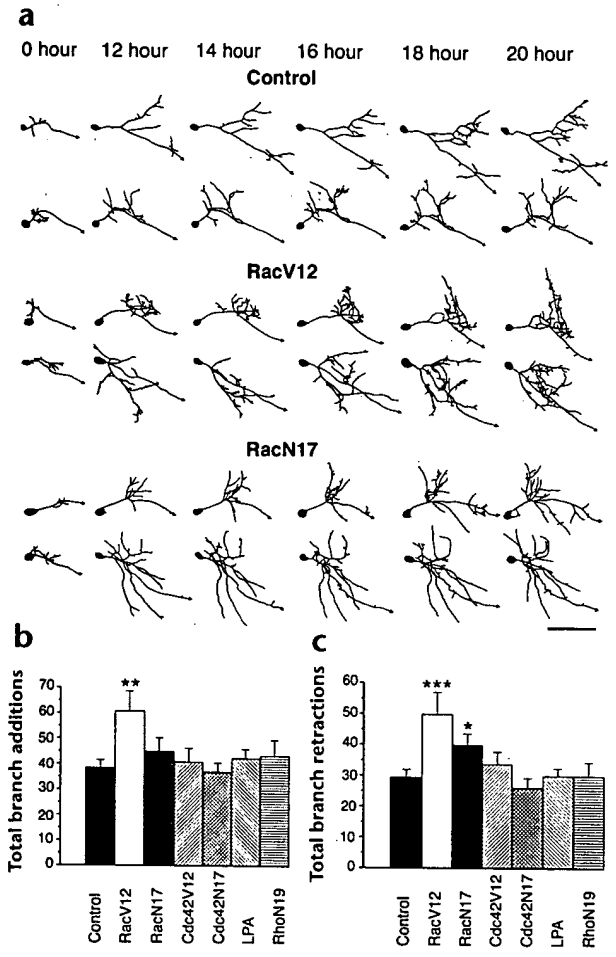


**Fig. 3.** Rac activity promotes dendritic branch dynamics. (**a**) Drawings of 2 representative tectal cells from control, constitutively active Rac virus-infected and dominant-negative Rac virus-infected animals imaged over 20 h. Note the rapid changes in fine branch tips in cells expressing constitutively active Rac. (**b**, **c**) Total branch additions (**b**) and retractions (**c**) for neurons imaged every two hours over eight hours. For each condition, 18–47 cells were analyzed. Scale bar, 50 μm. $*p < 0.05$; $**p < 0.002$; $***p < 0.001$.

of the arbor is very dynamic, characterized by continuous branch additions and retractions (Fig. 3). In animals infected with constitutively active Rac or dominant-negative Rac vaccinia virus, the dendritic arbor was more dynamic than in control neurons. As a result of these dynamics, the arbor structure was quite different from one time point to the next. Images of neurons infected with vaccinia virus expressing constitutively active Cdc42, dominant-negative Cdc42 and dominant-negative RhoA or neurons exposed to LPA did not show obvious changes in arbor dynamics during the eight-hour imaging period (see Fig. 3).

To quantify whether an increase or decrease in RhoA, Rac or Cdc42 activity had significant effects on arbor dynamics, we counted the numbers of newly added branches and retracted branches that were imaged during the eight-hour period. All groups had a comparable number of branch tips and total dendritic branch length at the first imaging observation. Neurons from animals infected with constitutively active Rac vaccinia virus added significantly more dendritic branches than control cells ($60.9 \pm 7.8$, constitutively active Rac; $38.5 \pm 3.0$, control, $p < 0.002$; Fig. 3). Constitutively active Rac cells also retracted significantly more dendrites than controls ($49.9 \pm 7.1$, constitutively active Rac; $29.4 \pm 2.5$, control; $p < 0.001$). Because constitutively active Rac enhanced both additions and retractions, the final number of branch tips was not significantly different from control cells. In contrast, dominant-negative Rac did not significantly alter rates of branch additions. Expression of dominant-negative Rac, however, did cause a significant increase in branch retractions compared to controls, but to a lesser extent than constitutively active Rac ($39.8 \pm 3.7$, $p < 0.05$). Experiments were also done using constitutively active Cdc42, dominant-negative Cdc42, dominant-negative RhoA and LPA. Comparable analysis indicated that neither Cdc42 nor RhoA affected branch dynamics (Fig. 3).

This analysis demonstrated that Rac activity affects rates of branch additions and retractions and suggested that the branches may be more transient. To test directly whether Rac, Cdc42 or RhoA alters the fate of dendritic branches, we designed an analysis to identify changes in the proportion of transient branches in an arbor. We divided all dendritic branches into four categories: stable branches, lost branches, new branches and transient branches (Fig. 4a). Stable branches are branches that are present throughout the imaging period. Lost branches are those that are present at the first image, but retracted over the eight-hour period. Any branches that were added after the first image and were still there at the last time point were categorized as new branches. The branches that were added after the first image and then retracted before the last image were categorized as transient branches. This analysis showed that among all the branches ever present during imaging, $34.8 \pm 1.2\%$ of them were transient in control cells. This number was significantly increased by the expression of constitutively active Rac, and to a lesser extent by constitutively active Cdc42 (**Table 1**; Fig. 4). Notably, dominant-negative Rac triggered an increase in transient branches (Fig. 4). In addition, dominant-negative Rac significantly decreased the relative number of new branches, that is, those that were added to the arbor and maintained to the end of the observation period. When we evaluated the fraction of stable branches, we noticed that only constitutively active Rac significantly affected this category, causing a 50% reduction of stable branches. No effects on arbor dynamics were observed when we expressed dominant-negative Cdc42 or dominant-negative RhoA or added LPA. Taken together, the analysis of arbor dynamics indicated that Rac and to a lesser extent Cdc42, but not RhoA are crucial in regulating arbor dynamics.

To test whether constitutively active Rac increases arbor dynamics in the time frame of minutes, we imaged single labeled neurons every 3 minutes over periods up to 30 minutes. Six cells were imaged from animals infected with either EGFP vaccinia virus (control) or constitutively active Rac vaccinia virus. The rapid branch dynamics are most easily recognized in a time-lapse movie of the cells (see http://neurosci.nature.com/web_specials/). The movie also demonstrates that dendritic arbors of neurons from animals infected with constitutively active Rac vaccinia virus appear more dynamic. Branches within an arbor can have a variety of lifetimes, ranging from several minutes to several days[3,4,23,24]. The lifetimes of branches can be shifted to longer or shorter times during development[4], by increased CaMKII activity[3] or by blocking NMDA receptor activity[6]. Quantitation of the lifetimes of each branch in the arbors show that 67% of branches in constitutively active Rac neurons have lifetimes less than 9 minutes, whereas only 31% of branches in control neurons have similarly short lifetimes. The shift toward shorter

branch lifetimes observed with expression of constitutively active Rac is consistent with the increase in the fraction of transient branches observed with the two-hour imaging protocol (Fig. 4). These data are consistent with the hypothesis that Rac affects cytoskeletal stability in neurons. Furthermore, they suggest that Rac affects arbor growth by affecting the rates of branch additions and retractions.

### GTPases affect arbor complexity

The constitutively active Rac neurons shown in Fig. 3 appear to have more densely branched dendritic arbors than control neurons. We used Sholl analysis, in which the number of branches crossing concentric rings around the cell body are counted (see Methods) to analyze dendritic arbor complexity. Expression of constitutively active Rac significantly altered dendritic arbor complexity compared to controls (Fig. 5). The distal dendritic arbors of neurons from animals infected with constitutively active Rac vaccinia virus were more complex than in controls, whereas proximal dendritic arbors were significantly less complex than in controls. Sholl analysis also confirmed the impression from the drawings in Fig. 3 that decreased RhoA activity enhanced arbor complexity, whereas LPA treatment caused simpler dendritic arbors.

### RhoA is involved in NMDAR-mediated arbor growth

We have previously shown that NMDA receptor activity is required for normal dendritic arbor elaboration in tectal neurons[5,6]. Normal arbor elaboration is prevented by exposing tectal neurons to the NMDA receptor antagonist 3-amino-phosphonovaleric acid (APV) early during dendritic arbor development[5], when the glutamatergic synaptic transmission from the retina is mediated predominantly by the NMDA receptor[25]. By contrast, the stability of dendritic arbors is less affected by APV in more mature neurons, when their retinotectal glutamatergic transmission is mediated predominantly by the AMPA-type glutamate receptor[25]. Here we report that increasing RhoA GTPase activity inhibits dendritic arbor development, whereas decreasing RhoA activity enhances dendritic growth. These data suggest that signals that decrease endogenous RhoA activity promote dendritic branch elongation. An intriguing model is that NMDA receptor activity may promote dendritic arbor development by decreasing endogenous RhoA activity. To test this possibility, we determined whether expression of dominant-negative RhoA could prevent the reduced dendritic growth rate observed as a result of blocking the NMDA receptor. As reported previously[5], exposing animals to 100 μM APV in their rearing solution inhibits dendritic arbor growth compared to control neurons (APV, 160 ± 15 μm per 20 h; control, 214 ± 17 μm per 20 h; $p < 0.05$; Fig. 6). Cells from animals infected with dominant-negative RhoA and treated with APV grow at 256 ± 32.5 μm per 20 h, which is significantly faster than in APV-treated neurons ($p < 0.005$), and comparable to neurons infected with dominant-negative RhoA vaccinia virus ($p = 0.55$). These data support a model in which NMDA receptor-mediated control of dendritic arbor elaboration operates through a pathway that decreases RhoA activity.

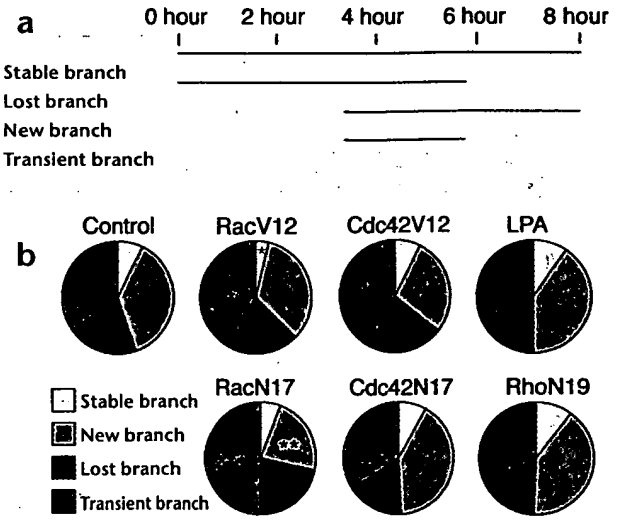


**Fig. 4.** Rac and Cdc42 increase transient branches. (a) Schematic drawing of the types of branch categories analyzed (see text for details). (b) Plots of the fraction of stable, transient, new and lost branches in arbors from each of the designated treatments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

## Discussion

The structure of the neuronal dendritic arbor determines the inputs received by the neuron as well as its integrative properties[1,2]. Children with mental retardation have severely reduced dendritic arbors, demonstrating a fundamental connection between neuronal structure and cognitive ability[26]. Consequently, mechanisms

**Table 1. Relative distribution of branches in each of the categories shown in Fig. 4.**

| | Stable branch (Percent total) | New branch (Percent total) | Lost branch (Percent total) | Transient branch (Percent total) |
|---|---|---|---|---|
| Control ($n = 47$) | 7.3 ± 0.8 | 37.6 ± 1.7 | 20.3 ± 1.4 | 34.8 ± 1.3 |
| RacV12 ($n = 20$) | 3.7 ± 1.1 $p < 0.013$ | 34.1 ± 1.7 | 19.5 ± 1.8 | 42.7 ± 2.3 $p < 0.002$ |
| RacN17 ($n = 18$) | 5.7 ± 1.0 | 22.8 ± 7.8 $p < 0.008$ | 21.8 ± 2.7 | 49.7 ± 5.5 $p < 0.001$ |
| Cdc42V12 ($n = 22$) | 7.4 ± 1.4 | 28.2 ± 6.5 | 22.0 ± 1.9 | 42.4 ± 4.2 $p < 0.03$ |
| Cdc42N17 ($n = 21$) | 8.4 ± 1.3 | 40.9 ± 2.3 | 16.5 ± 1.9 | 34.2 ± 2.1 |
| LPA ($n = 27$) | 9.3 ± 0.9 | 40.6 ± 2.2 | 18.5 ± 1.3 | 31.6 ± 2.2 |
| RhoN19 ($n = 20$) | 10.4 ± 1.4 | 40.1 ± 2.4 | 16.4 ± 1.4 | 33.2 ± 2.2 |

Values for numbers of cells analyzed also apply to data in Figs. 2, 3 and 5.

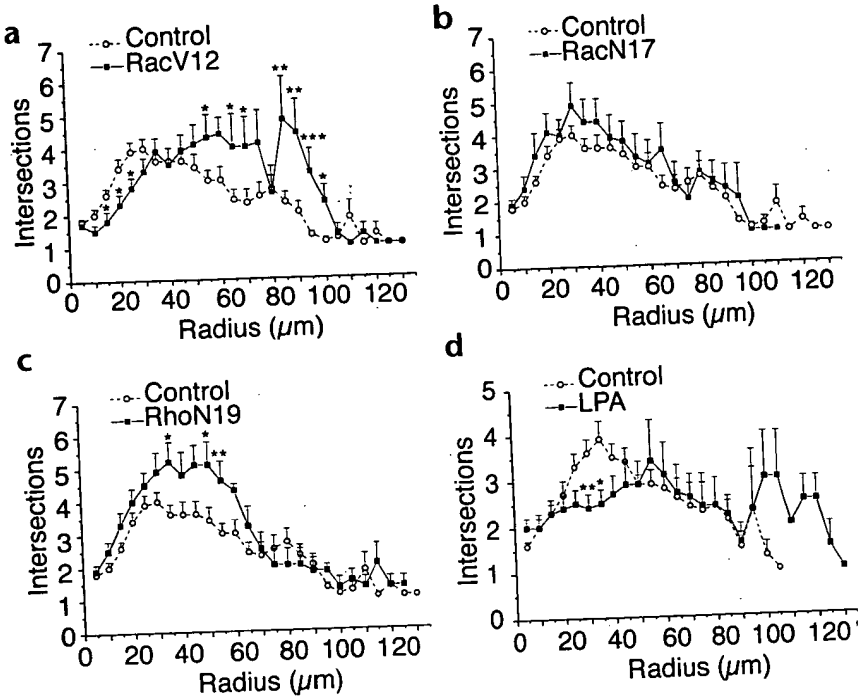


**Fig. 5.** Rac and RhoA affect dendritic arbor complexity. Sholl analysis of constitutively active Rac (**a**), dominant-negative Rac (**b**), dominant-negative Rho (**c**) and LPA-treated (**d**) neurons compared to controls. Expression of constitutively active Rac significantly decreases the number of dendritic branches close to the cell body, but significantly increases arbor complexity distal to the cell body. Decreasing RhoA activity increases arbor complexity, whereas increasing RhoA activity decreases complexity. *$p < 0.05$; **$p < 0.001$; ***$p < 0.0001$.

that influence the development of neuronal structure are likely to significantly affect brain function. Mutations in a Rho-GTPase activating protein are found in patients with X-linked mental retardation[27], suggesting that GTPase signaling is required for the development of normal brain function.

Here we have shown that different members of the Rho family of GTPases regulate different aspects of dendritic arbor elaboration in the intact animal. By taking sequential images of individual dendritic arbors with the confocal microscope, we found that Rac, and to a lesser extent Cdc42, regulate branch dynamics. RhoA specifically controls branch elongation, without directly affecting branch dynamics. These data suggest that dendritic arbor elaboration occurs as a result of Rac-mediated branch dynamics followed by RhoA-mediated branch extension. Together the activities of the GTPases contribute to the net growth of dendritic arbors in the intact animal. Because many tectal neurons are infected and express ectopic GTPases in these experiments, an interesting possibility, which we cannot exclude, is that the observed effects of the GTPases are in part due to GTPase expression in cells other than the ones imaged. However, Rho affects dendritic arbor development in a cell-autonomous fashion in *Drosophila*[20].

### RhoA regulates dendritic branch extension

Exposure to LPA, an activator of RhoA, severely impairs dendritic arbor elaboration, whereas expression of dominant-negative Rho enhances dendritic arbor growth, consistent with the findings in neuronal cell lines, where LPA causes neurite retraction and inhibition of RhoA induces neurite outgrowth[10,11]. This suggests that endogenous RhoA activity in developing tectal neurons is high and that dendritic arbor growth is actively promoted under conditions that inhibit RhoA activity. Because neither LPA nor dominant-negative RhoA affects rates of branch additions or retractions, these experiments indicate that RhoA selectively affects extension or retraction of existing branches.

We previously showed that blocking the NMDA type of glutamate receptor decreases the rate of branch additions and decreases the extension of existing branches during early stages of dendritic arbor formation[5,6]. Later, as the neurons mature, their dendritic arbor structure becomes more stable. These mature neurons express calcium/calmodulin-dependent protein kinase (CaMKII), and enhanced CaMKII activity decreases rates of dendritic branch additions and retractions[3]. One intriguing scenario is that glutamatergic synaptic activity may promote dendritic arbor elaboration by decreasing endogenous RhoA activity in dendrites of immature tectal cells. Indeed, we find that the decreased arbor growth rate observed when NMDA receptors are blocked is counteracted by expression of dominant-negative RhoA. These data suggest a mechanism in which RhoA activity and dendritic branch extension may be controlled locally by synaptic inputs. Furthermore, as neuronal structure matures and becomes more stable, glutamatergic synaptic inputs and CaMKII activity may operate through Rac and Cdc42 to control structural plasticity. The demonstration of a Ras GTPase-activating protein that is regulated by NMDA receptor and CaMKII activity[28,29] combined with evidence of crosstalk between Ras and Rac in controlling cell morphology[30] further suggest that such a regulatory pathway may exist.

### Rac regulates dendritic arbor dynamics

Data on the function of Rac in regulating dendritic arbor structure in the intact animal is limited[16,18,31]. In *Drosophila* and mice, Rac does not affect the overall development of dendritic morphology[15,16], whereas in *Xenopus* retinal cells, Rac does affect dendritic arbor outgrowth[18]. Our data show that Rac is involved in the formation and turnover of dendrites, but not dendritic branch extension. At a single time point, the total number and length of dendrites in animals expressing constitutively active or dominant-negative Rac are comparable to the control values, but time-lapse imaging clearly demonstrates that constitutively active Rac increases branch additions and retractions. The final branch numbers and total branch length are the same as control neurons because enhanced rates of branch additions are balanced by increased branch retractions. One conceivable interpretation for the constitutively active Rac phenotype is that increased Rac activity triggers the initiation of dendritic branches, but Rac activity alone is not sufficient to maintain the newly formed branches. Consequently, they are retracted. Rac activity is also required to maintain dendritic branches, because more dendrites are retract-

ed when Rac activity is reduced by expression of dominant-negative Rac. In neurons from animals infected with dominant-negative Rac vaccinia virus, the enhanced rates of branch retractions contribute to the increased fraction of transient branches in these arbors. These experiments indicate the increased Rac activity promotes branch additions. They further suggest that Rac activity is necessary but not sufficient for branch maintenance.

In addition to regulating the rate of branch additions, Rac is also involved in shaping the architecture of the dendritic arbor. Sholl analysis indicates that enhanced Rac activity increases the overall complexity of the dendritic arbor. Constitutively active Rac increased the complexity of the distal dendritic arbor, whereas it decreased the complexity of the proximal dendritic tree. The elaboration of proximal and distal dendrites are differentially regulated[2], possibly as a result of differences in lamina-specific input activity and the distributions of environmental signals such as adhesion molecules[32] and neurotrophins[33]. Indeed, GTPase activity has been suggested to differentially regulate the elaboration of basal and apical dendrites in dissociated cortical neurons[12]. Because the Rho family GTPases are regulated by extracellular stimuli[9], and also seem to mediate neuronal responses to substrate-bound guidance cues, including myelin and integrins[34–36], normal dendritic arbor elaboration may reflect the complex composition of environmental cues in intact animals. Consequently, in our experiments, introduction of constitutively active Rac may have had different outcomes in distal and proximal dendrites because of local variations in endogenous GTPase activity within the arbor.

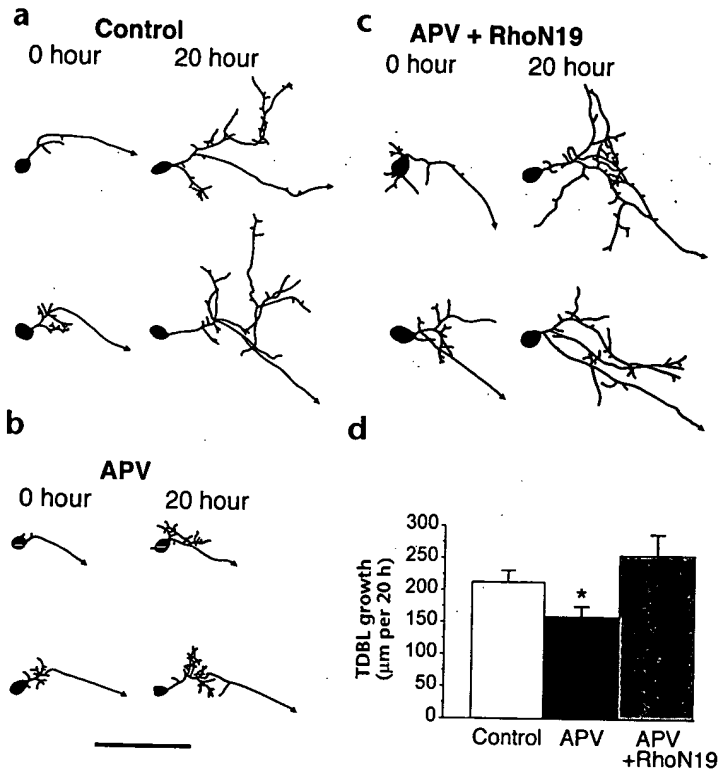


**Fig. 6.** NMDA receptor-mediated arbor growth operates through RhoA. Drawings of two representative neurons from controls (**a**), APV-treated animals (**b**) and APV-treated animals expressing dominant-negative Rho (**c**). (**d**) Change in total dendritic branch length (TDBL) for neurons from the designated groups. For each condition, 12–24 neurons were analyzed. Scale bar, 50 μm. *$p < 0.05$.

Increased Cdc42 activity in tectal neurons increased the proportion of transient dendrites. Active Cdc42 induces filopodia in growth cones[37], which serve a sensory role in axon pathfinding. Cdc42 may serve a similar function to promote filopodia in the dendritic arbor, but does not seem to regulate either the stabilization or extension of dendritic branches.

## Interaction of GTPases with the cytoskeleton

Our data suggest that the GTPases modify specific aspects of dendritic arbor morphology. Rac seems to govern the rates of additions and retractions of new branches without affecting subsequent regulatory events that determine whether the branch will extend. The increased actin filaments we observed by phalloidin staining in constitutively active Rac and constitutively active Cdc42 cells suggest that these GTPases likely mediate their effects on the dendritic arbor dynamics by affecting the actin cytoskeleton. Although constitutively active Cdc42 expression increased filamentous actin according to the phalloidin staining, it had only a modest effect on the parameters of dendritic dynamics assayed here. It is possible that Cdc42 affects different aspects of cytoskeletal structure in neurons that were not assessed in this study. Neither LPA nor dominant-negative RhoA had a significant effect on branch dynamics, although they clearly regulated branch shortening and extension, respectively. This suggests that RhoA activity influences the extension or retraction of existing branches but does not govern the emergence of new branches. Addition and retraction of short branches, maintenance of branches and their extension are distinct cell biological events that are likely controlled by different regulatory mechanisms, with respect to the cytoskeleton. The cytoskeleton of transient dendritic branches may be entirely actin-based[38] and their addition likely due to actin polymerization[39]. The maintenance of a newly added branch may be due to the invasion of the new branch by microtubules[39], as described for growth cones[40]. Finally branch extension may be due to assembly of microtubules. Although all three GTPases are reported to regulate the actin cytoskeleton, RhoA may also regulate tubulin assembly[41], supporting the idea that RhoA's principal effect on dendritic arbor elaboration is through the extension or retraction of existing dendritic branches, which are enriched in microtubules[38]. In addition, GTPases can regulate cell–cell contacts through presentation and clustering of cadherins and integrins on the cell surface[7]. Evidence that cadherins can function in synapse stabilization[42] suggests an intriguing connection between GTPases and synaptogenesis.

The GTPases are positioned in the midst of several signal transduction pathways, which govern cell shape and polarity[7,9]. The activity of each GTPase may be altered downstream of cell surface receptors, including growth factors, cytokines and neurotransmitters[7,14,37]. We provide evidence that the NMDA-type glutamate receptor may be upstream of the RhoA GTPase in regulating dendritic arbor elaboration. Indeed, it seems that NMDA receptor activity decreases RhoA activity and thereby increases branch elongation. This observation is surprisingly complementary to a report that activation of the p75 neurotrophin receptor can increase retinal axon elongation by decreasing endogenous RhoA activity[14]. In addition, each of the GTPases

affects the cytoskeleton through downstream effectors. It would be of interest to identify the interacting partners through which the GTPases regulate dendritic arbor growth. Potential downstream candidates for RhoA are the serine/threonine kinase, ROCK, and mDia, which regulate the formation of different types of actin fibers[43]. Rac may act through LIM kinase and cofilin[44,45]. Filopodia formation induced by Cdc42 seems to depend on N-WASP, a ubiquitously expressed Cdc42 binding protein[46]. Finally, Rac and Cdc42 share common effectors, including the serine/threonine kinase PAK, which is involved in the morphological changes mediated by Rac and Cdc42 (refs. 9, 47).

## Methods

**Construction of recombinant vaccinia virus.** Human RacV12, RacN17 and Cdc42V12 were myc tagged at the amino (N) terminal. Myc-RacV12, myc-RacN17 and myc-Cdc42V12 were cloned into the *Sal* I/*Spe* I site upstream of IRES-EGFP in the pBluescript vector. The myc-GTPase-IRES-EGFP constructs were cut out from pBluescript vector and cloned into the vaccinia virus vector pSC65 downstream of a strong synthetic early/late vaccinia virus promoter. The cDNA encoding EGFP-Cdc42N17 and EGFP-RhoN19 N-terminal fusion proteins were cloned into the *Sal* I /*Sma* I sites of pSC65 downstream of the strong synthetic early/late vaccinia virus promoter. The EGFP fusion proteins are active *in vitro* in a variety of cell lines assayed for cell morphology and cell adhesion (L. Van Aelst, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, personal communication). A virus expressing only EGFP driven by the early/late promotor was used as a control for the effects of viral infection. All viruses also express β-galactosidase (β-gal) behind a weaker p7.5 viral promotor, which is used for plaque selection of recombinant viruses[48]. Constructs were confirmed by sequencing. Recombinant vaccinia viruses, obtained by homologous recombination of pSC65 and wild-type vaccinia virus as reported[3], were purified and titered before use[48]. High-titer virus (over $10^7$ plaque-forming units, pfu) was used to infect animals in imaging experiments.

**Viral infection.** Albino *Xenopus* laevis tadpoles were obtained by mating induced by human chorionic gonadotropin injections. Purified virus (100–150 nl mixed with 0.1% fast green) was injected into the tectal ventricle of stage 46 tadpoles anesthetized with 0.02% 3-aminobenzoic acid ethyl ester (MS222).

**Immunostaining.** Animals were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH7.4) overnight at 4°C, rinsed in PB and cryoprotected in 30% sucrose before brains were cut into 20-μm cryostat sections. For myc immunostaining, sections were preincubated with blocking solution containing 5% goat serum and 0.3% Triton X-100 in PB for 1 h, followed by an overnight incubation at 4°C in anti-myc antibody (Calbiochem), diluted 1:100 in blocking solution. After rinsing, sections were incubated in cy5-goat anti-mouse secondary antibody (diluted 1:100 in blocking solution; Jackson Immunoresearch Laboratories, West Grove) for 30 minutes. We did not observe differing levels of myc immunoreactivity in neurons, suggesting that expression levels of the GTPases did not vary over a detectable range. Rhodamine-phalloidin (Molecular Probes Eugene, Oregon; final dilution 1:800) was added to the incubation solution together with secondary antibody for double labeling.

**Image acquisition.** Single tectal neurons were labeled by iontophoresis of DiI (0.02% 1,1′-dioctadecyl-3,3,3′3′-tetramethylindocarbocyanine perchlorate, Molecular Probes) in ethanol. Positive current (1–10 nA) was used in 3–10 pulses of 200-ms duration. In animals infected with EGFP fusion proteins or IRES-EGFP constructs, DiI-labeled neurons were located within an infected region of the tectum; however, it was not always possible to verify that each DiI-labeled neuron was infected. We selected animals with single brightly labeled neurons two hours after DiI labeling. Images were collected at 2-μm steps through the entire *z* dimension of labeled neurons with a Noran Instruments XL laser scanning confocal attachment mounted on an upright Nikon Optiphot through a 40× Nikon oil immersion lens (1.30 NA). Each optical section was an average of 8–16 frames. Animals were anesthetized with 0.02% MS222 during DiI labeling, screening and imaging. Animals recovered from anesthesia between imaging sessions, except for the three-minute imaging experiment, where animals were anesthetized throughout the imaging protocol. In experiments using LPA to activate RhoA or APV to block NMDA receptors, the drug was added to the rearing solution immediately after the first image was collected.

**Image analysis.** Dendritic arbors were reconstructed by tracing the portion of the neuron in each optical section onto an acetate sheet until the entire neuron was drawn. This method provides a more detailed representation of the morphology than the three-dimensional reconstruction generated by computer, because fine processes visible in the optical sections are lost in the computer-generated reconstruction. Total dendritic branch length was measured from scanned drawings of cells with NIH Image 1.61. The number of branch tips was counted manually. To analyze the arbor dynamics, drawings of cells from sequential time points were superimposed to identify added and retracted branches. The magnitude of arbor dynamics determined in this and previous studies[3,4], in terms of numbers of branches added or retracted over a two-hour period, may be underestimated by five- to tenfold[24,49], because unobserved branches are both added and retracted during the two-hour intervals. The shortest interval over which we can collect images is three minutes because this is about the time it takes to collect and save a single *z* series through the optic tectum. Observations collected at such frequent time points over our 20-hour imaging period would provide a more accurate value of branch additions and retractions; however, neither the DiI-labeled neurons nor the animals can survive such prolonged imaging session or sustained anesthesia[49]. Branch additions, branch retractions and the change in total dendritic branch length in uninfected control animals and animals infected with EGFP vaccinia virus were comparable, consistent with previous observations that vaccinia virus does not affect the development of tectal cell morphology[3]. We therefore pooled these two groups of control cells together.

Sholl analysis was done with Object-Image software (NIH Image). A scanned drawing of the neuron was overlaid on a series of concentric circles, spaced every 5 μm with the cell body in the center. The number of dendritic branches crossing each concentric circle was marked on the composite image and counted.

Statistical analysis was done with two-tailed *t*-test.

*Note: Time-lapse movies can be found on the Nature Neuroscience web site (http://neurosci.nature.com/web_specials/).*

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